Remarks

Reconsideration of this Application is respectfully requested.

I. Status of the Claims

Claims 45-49 and 52 are pending in the application, with claim 45 being the sole independent claim.

II. Summary of the Office Action

In the Office Action dated January 15, 2002, the Examiner has made three rejections of the claims, two rejections under 35 USC § 102 and one rejection under 35 USC § 103. Based on the following remarks, Applicants respectfully request that the Examiner reconsider all outstanding rejections and that they be withdrawn.

III. Claim Rejections Under 35 USC § 102

A. Chenchik

The Examiner has rejected claims 45-47, 49 and 52 under 35 USC § 102(e) as being anticipated by Chenchik *et al.*, U.S. Patent No. 5,565,340 ("Chenchik"). *See* Paper No. 10, page 2, item 3. Applicants respectfully traverse this rejection.

According to the Examiner:

> Chenchik et al teach restriction digestion of PCR products prepared using a DNA polymerase that was inactivated with anti-DNA polymerase, and thereby teach compositions comprising restriction endonucleases and polymerase inhibitors (col 20, lines 38-67; col 21, lines 1-32).

Paper No. 10, page 2. Applicants respectfully disagree with this assessment.

Under 35 USC § 102, a claim can only be anticipated if every element in the claim is expressly or inherently disclosed in a single prior art reference. *See Kalman v. Kimberly Clark Corp.*, 713 F.2d 760, 771 (Fed. Cir. 1983), *cert. denied*, 465 U.S. 1026 (1984); *see also PPG Industries, Inc. v. Guardian Industries Corp.*, 75 F.3d 1558, 1566 (Fed. Cir. 1996) ("[t]o anticipate a claim, a reference must disclose every element of the challenged claim and enable one skilled in the art to make the anticipating subject matter.") Applicants respectfully assert that Chenchik does not teach, expressly or inherently, all of the elements of Applicants' claims, and therefore does not support a rejection under § 102.

Claim 45- 49 and 52 are directed to compositions comprising one or more restriction endonucleases and one or more polymerase inhibitors. A polymerase inhibitor is defined in the specification as "any compound, composition or combination thereof that inactivates or reduces the activity of a polypeptide having nucleic acid polymerase activity, reversibly or irreversibly." *See* specification at page 35, lines 21-23. As discussed in more detail below, Chenchik does not teach a composition comprising one or more restriction endonucleases and one or more polymerase inhibitors as this latter term is defined in the specification.

Chenchik describes the addition of TthSTART antibody to a Tth/Vent enzyme mixture. *See* Chenchik at column 21, lines 6-9. The Tth/Vent enzyme/TthSTART antibody mixture is then used in a PCR reaction. *See* Chenchik at column 21, lines 10-14. The cycle parameters of the PCR reaction are as follows:

> *denaturation at 94°C* for 30 seconds and annealing/extension at 68°C for 6 minutes except that *the first denaturation step was for 1 minute* and the final annealing/extension time was

> lengthened to 13 minutes. Thirty (30) cycles of primary PCR were typically used.

See Chenchik at column 21, lines 15-20 (emphasis added). A second PCR reaction was conducted using the same reaction parameters as above except that 20 cycles were used. *See* Chenchik at column 21, lines 21-26. Finally, the PCR products were mixed with enzyme buffer and restriction enzyme and incubated at 37°C for 4 hours. *See* Chenchik at column 21, lines 28-32. As explained below, this mixture does not contain a polymerase inhibitor; that is, it does not contain a "compound, composition or combination thereof that inactivates or reduces the activity of a polypeptide having nucleic acid polymerase activity, reversibly or irreversibly," and therefore does not fall within the scope of Applicants' claims.

It is well known in the art that anti-polymerase antibodies such as TthSTART function to block polymerase activity during the set-up of PCR reactions. *See* CLONTECH TthStart Antibody User Manual (March 2000) (hereinafter, "the TthStart Manual," copy submitted herewith as Exhibit A) at page 3. Importantly, the TthStart antibody is rendered non-functional when the temperature is raised above 70°C.

> The inhibition of *Tth* DNA polymerase is completely reversed when the temperature is raised above 70°C. *At the first template-denaturation step in thermal cycling, the enzyme-antibody complex dissociates and the TthStart Antibody is rendered nonfunctional.* At the same time, the activity of the *Tth* DNA polymerase is restored, and the enzyme functions normally during the course of the PCR.

TthStart Manual at page 3 (emphasis added).

Therefore, in the procedure that is set forth in Example 2 of Chenchik, the TthSTART antibody is rendered nonfunctional at the first denaturation step when the temperature is raised to 94°C for 1 minute. The *nonfunctional* TthSTART antibody is not

a "polymerase inhibitor," as this term is defined in the specification. Thus, at the conclusion of the PCR, when restriction enzyme is added to the PCR reaction mixture, the PCR reaction mixture does not contain a "polymerase inhibitor;" *i.e.*, the PCR reaction mixture does not contain "any compound, composition or combination thereof that inactivates or reduces the activity of a polypeptide having nucleic acid polymerase activity." Chenchik accordingly does not teach a composition comprising one or more restriction endonucleases and one or more polymerase inhibitors.

With respect to claim 49, the Examiner stated that:

> it is an inherent property of the compositions taught by Chenchik et al that they are "stable upon storage" (e.g., such compositions would be stable when frozen). Further, it is noted that Chenchik et al teach providing restriction endonucleases and polymerase inhibitors in kits, and thereby exemplify reagents that are "stable upon storage", as required by the instant claim (col 11, lines 10-24).

Paper No. 10, pages 2-3.

Applicants respectfully disagree with this contention. First, as discussed above, the composition disclosed in Chenchik does not comprise one or more restriction endonucleases and one or more polymerase inhibitors since the TthSTART antibody is nonfunctional at the point at which endonuclease is combined with the PCR reaction mixture. Second, Applicants remind the Examiner that "[i]n order for a disclosure to be inherent . . . the missing descriptive matter must necessarily be present in the [cited reference] such that one skilled in the art would recognize such a disclosure." *Tronzo v. Biomet, Inc.*, 156 F.3d 1154, 1159 (Fed. Cir. 1998). Moreover, "the examiner must provide a basis in fact and/or technical reasoning to reasonably support the determination that the allegedly inherent characteristic *necessarily* flows from the teachings of the applied prior art." *Ex parte Levy*,

17 USPQ2d 1461, 1464 (PTO Bd. Pat. App. Int. 1990) (emphasis in original). The Examiner has pointed to nothing in Chenchik to indicate that stability upon storage is *necessarily* an inherent property of the compositions taught in Chenchik. Hence, the Examiner's attempted reliance upon inherent anticipation in the present case is factually and legally unfounded. Third, Applicants note that the individual components of the kits described in Chenchik at column 11, lines 10-24, are described as being "in separate packaging or compartments." *See* Chenchik at column 11, line 11. Thus, the kits described in Chenchik do not include a composition comprising one or more restriction endonucleases and one or more polymerase inhibitors, wherein said restriction endonucleases and/or polymerase inhibitors are stable upon storage. Therefore, Chenchik cannot and does not anticipate claim 49.

Since Chenchik does not disclose, either explicitly or inherently, all of the elements of Applicants' claims, Applicants respectfully request that the rejection of claims 45-47, 49 and 52 under 35 USC § 102(e), as being anticipated by Chenchik, be reconsidered and withdrawn.

B. Okura

The Examiner has also rejected claims 45-49 and 52 under 35 USC § 102(e) as being anticipated by Okura *et al.*, U.S. Patent No. 6,060,283 ("Okura"). *See* Paper No. 10, pages 3-4, item 4. Applicants respectfully traverse this rejection.

According to the Examiner:

> Okura et al disclose a composition comprising TAQSTART antibody and the restriction enzymes *Hind*III and *Sph*I, and a composition comprising TAQSTART antibody and the

> restriction enzymes *Not*I and *Sph*I (Col 10, lines 11-51, particularly lines 33-35 and 48-50).

Paper No. 10, pages 3-4.

Applicants submit that Okura does not disclose a composition comprising one or more endonucleases and one or more polymerase inhibitors. Okura describes a PCR reaction containing TaqStart antibody. *See* Okura at column 10, lines 13-29. Following the addition of TaqStart antibody to the reaction mixture, the following reaction parameters were applied:

> After incubating at 94°C for one min, the mixture was subjected to 5 cycles of incubations at 98°C for 20 sec and at 72°C for 7 min, followed by 25 cycles of incubations at 98°C for 20 sec and 68°C for 7 min to perform PCR.

See Okura at column10, lines 29-33. Okura then states that the reaction mixture was cleaved by HindIII and SphI. *See* Okura at column 10, lines 33-36.

TaqStart antibody, like TthStart antibody, is used to block polymerase activity during the set-up of PCR reactions. *See* CLONTECH TaqStart Antibody User Manual (January 2001) (hereinafter, "the TaqStart Manual," copy submitted herewith as Exhibit B) at page 3. As with TthStart, TaqStart antibody is rendered non-functional when the temperature is raised above 70°C. *See* the TaqStart Manual at page 3.

Therefore, in the procedure that is set forth in Example 2 of Okura, the TaqStart antibody is rendered nonfunctional at the first denaturation step when the temperature is raised to 94°C for one minute. The *nonfunctional* TaqStart antibody is not a "polymerase inhibitor," as this term is defined in the specification. Thus, at the conclusion of the PCR, when reaction mixture is cleaved by restriction enzymes, the reaction mixture does not contain a "polymerase inhibitor;" *i.e.*, the reaction mixture does not contain "any compound,

composition or combination thereof that inactivates or reduces the activity of a polypeptide having nucleic acid polymerase activity." Okura accordingly does not teach a composition comprising one or more restriction endonucleases and one or more polymerase inhibitors.

With respect to claim 49, the Examiner stated that "it is an inherent property of the reagents taught by Okura et al and of the compositions taught by Okura et al that they are 'stable upon storage' (e.g., such compositions would be stable when frozen)." *See* Paper No. 10, page 4.

Applicants respectfully disagree with this contention. As discussed above, the compositions disclosed in Okura do not comprise one or more restriction endonucleases and one or more polymerase inhibitors since the TaqStart antibody is nonfunctional at the point at which endonuclease is combined with the reaction mixture. In addition, the inherency standards under *Tronzo* and *Levy* are not met by the disclosure of Okura, since the Examiner has pointed to nothing in Okura to indicate that stability upon storage is an inherent property of the compositions taught therein. Therefore, Okura cannot and does not anticipate claim 49.

Since Okura does not disclose, either explicitly or inherently, all of the elements of the present claims, Applicants respectfully request that the rejection of claims 45-49 and 52 under 35 USC § 102(e), as being anticipated by Okura, be reconsidered and withdrawn.

V. Claim Rejection Under 35 USC § 103

The Examiner has also rejected claim 48 under 35 USC § 103(a) as being unpatentable over Chenchik. *See* Paper No. 10, page 4, item 5. Applicants respectfully traverse this rejection.

In order to establish a *prima facie* case of obviousness, all of the claim limitations must be taught or suggested by the prior art. *See In re Royka*, 490 F.2d 981, 180 USPQ 580 (CCPA 1974). In addition, there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings. *See In re Rouffet*, 149 F.3d 1350, 1357, 47 USPQ2d 1453, 1457-58 (Fed. Cir. 1998). The teaching or suggestion to make the claimed combination must be found in the prior art, not in Applicants' disclosure. *See In re Vaeck*, 947 F.2d 488, 20 USPQ2d 1438 (Fed. Cir. 1991). In view of these legal prerequisites under § 103, Applicants respectfully assert that a *prima facie* case of obviousness cannot be established with respect to claim 48.

Claim 48 is directed to compositions comprising one or more restriction endonucleases and one or more polymerase inhibitors, *i.e.*, an antibody selected from the group consisting of an anti-*Taq* antibody, an anti-*Tne* antibody, an anti-*Tma* antibody, anti-*Pfu* antibody, and fragments thereof. A polymerase inhibitor is defined in the specification as "any compound, composition or combination thereof that inactivates or reduces the activity of a polypeptide having nucleic acid polymerase activity, reversibly or irreversibly." *See* specification at page 35, lines 21-23. Thus, in view of this definition, the antibodies that are included within the scope of claim 48 are only those that are capable of inactivating or reducing the activity of a polypeptide having nucleic acid polymerase activity.

Chenchik does not teach or suggest all of the elements of claim 48. Specifically, the reaction mixture to which endonuclease was added in Example 2 of Chenchik does not contain an antibody that is capable of inactivating or reducing the activity of a polypeptide having nucleic acid polymerase activity. *See* Applicants' discussion set forth in *Section*

III.A. above. Thus, even if a skilled artisan were somehow motivated to replace the TthSTART antibody used in Example 2 with one of the antibodies recited in claim 48, the resulting composition would not possess all of the attributes of claim 48, namely, the composition would not contain an endonuclease and a polymerase inhibitor. Accordingly, a *prima facie* case of obviousness cannot be established with respect to Applicants' claims. Applicants therefore respectfully request that the rejection of claim 48 under 35 USC § 103(a), as being unpatentable over Chenchik, be reconsidered and withdrawn.

Conclusion

All of the stated grounds of objection and rejection have been properly traversed, accommodated, or rendered moot. Applicants therefore respectfully request that the Examiner reconsider all presently outstanding objections and rejections and that they be withdrawn. Applicants believe that a full and complete reply has been made to the outstanding Office Action and, as such, the present application is in condition for allowance. If the Examiner believes, for any reason, that personal communication will expedite prosecution of this application, the Examiner is invited to telephone the undersigned at the number provided.

Prompt and favorable consideration of this Reply is respectfully requested.

Respectfully submitted,

STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C.

Frank R. Cottingham

Frank R. Cottingham
Attorney for Applicants
Registration No. 50,437

Date: 5/08/02

1100 New York Avenue, N.W.
Suite 600
Washington, D.C. 20005-3934
(202) 371-2600

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TthStart™ Antibody User Manual

PT1578-1 (PR02236)

Published 1 March 2000

Catalog #: 5401-1

See List of Components for storage conditions

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TaqStart and TthStart Antibodies are licensed under U.S. Patent No. 5,338,671 and corresponding patents in other countries.

I. Introduction

Long-distance PCR (LD PCR) procedures have been developed that use a mixture of either *Taq* or *Tth* polymerase and a small amount of another thermostable DNA polymerase possessing 3'–5' exonuclease "proofreading" activity (Barnes, 1994; Cheng *et al.*, 1994). We have found that the "hot start" technique is often needed to give the desired results in LD PCR, particularly for amplification of single copy genes from genomic DNA.

Hot start PCR is commonly used to enhance the specificity and sensitivity of PCR (D'Aquila *et al.*,1991; Chou *et al.*, 1992; Faloona *et al.*, 1990). In many cases, hot start PCR produces greater yields of single products than is possible with conventional PCR. However, manual hot start PCR is inconvenient, time-consuming, and incurs a risk of cross-contamination. TthStart™ Antibody makes it possible to perform "TthStart" PCR, with all the advantages—and none of the disadvantages—of hot start PCR.

TthStart Antibody is a neutralizing monoclonal antibody to DNA polymerase of *Thermus thermophilus* (Findlay *et al.*, 1993; Sharkey *et al.*, 1994). Used in a manner similar to TaqStart™ Antibody (Kellogg *et al.*, 1994), TthStart Antibody blocks polymerase activity during set-up of PCR reactions at ambient temperatures (20–22°C). The inhibition of *Tth* DNA polymerase is completely reversed when the temperature is raised above 70°C. At the first template-denaturation step in thermal cycling, the enzyme-antibody complex dissociates and the TthStart Antibody is rendered nonfunctional. At the same time, the activity of *Tth* DNA polymerase is restored, and the enzyme functions normally during the course of the PCR. TthStart Antibody is effective with a variety of commercially available *Tth* DNA polymerases (native and recombinant). However, the antibody is not suitable for use with N-terminal deletions of *Tth* enzyme (e.g., Δ*Tth*).

The use of TthStart Antibody significantly enhances the results of PCR experiments which can be improved by a hot start because TthStart PCR reduces generation of nonspecific PCR products and primer-dimer artifacts that compete with the desired product of PCR. As a result, a greater yield of the specific product is possible with TthStart PCR. For use in LD PCR up to 40 kb, TthStart Antibody is also available in Advantage® Genomic Polymerase Mix (#8418-1), a mixture of *Tth* DNA polymerase, a small amount of a proofreading enzyme, and TthStart Antibody. Advantage Genomic Polymerase Mix is also available as part of the Advantage® Genomic PCR Kit (#K1906-1).

II. List of Components

Store components at –20°C. Avoid the use of "frost-free" freezers.

The amounts provided are sufficient for 200 reactions.

5401-1 (200 reactions):

- 80 µl **TthStart™ Antibody** (14 µM; 2.2 µg/µl) in storage buffer: 10 mM Tris-HCl (pH 7.0), 50 mM KCl, 50% glycerol
- 1.0 ml **Dilution buffer** 10 mM Tris-HCl (pH 7.0), 50 mM KCl

III. Additional Materials Required

The following materials are required but not supplied.

- ***Tth* DNA polymerase** *
- **10X Tth reaction buffer** (avoid the use of buffers containing formamide)
- **$Mg(OAc)_2$** (25 mM)
- **dNTP mixture** (dATP, dCTP, dGTP, & dTTP; 10 mM final concentration of each dNTP in mixture; #4700-1)
- **Sterile, distilled water**

* CLONTECH's *Tth* polymerase mix, Advantage® Genomic Polymerase Mix (#8418), already contains TthStart Antibody.

IV. TthStart Protocol

PLEASE READ ENTIRE PROTOCOL BEFORE STARTING.

A. General Considerations

1. Storage and use

   In the supplied storage buffer (containing 50% glycerol) and at the supplied concentration (>1 µg/µl), TthStart Antibody will not freeze at –20°C. Repeated freezing and thawing of diluted TthStart Antibody may adversely affect its function. There are two basic approaches to using TthStart Antibody which avoid repeated freeze/thaw cycles:

   a. If you wish to retain the option of using different molar ratios of TthStart Antibody to *Tth* DNA polymerase in different experiments, dilute a portion of the TthStart Antibody to a working concentration and mix with *Tth* DNA polymerase immediately before use (Section IV.B). The dilution may be stored at 4°C for ≤3 months, and the undiluted portion of the TthStart Antibody at –20°C.

   b. For convenience if you plan to use the same molar ratio of TthStart Antibody to *Tth* DNA polymerase for several experiments, add undiluted TthStart Antibody to a batch of *Tth* DNA polymerase, aliquot, and store at –20°C for later use (see Section IV.C).

2. Choice of enzymes

   TthStart Antibody binds to and inactivates DNA polymerase of *Thermus thermophilus*. It does not bind to *ΔTth* DNA polymerase (TOYOBO). It may be necessary to titrate the TthStart Antibody (relative to the polymerase) before starting any experiments (Section V.B). TthStart is not suitable for use with *Taq* DNA polymerase.

3. Use of additives

   TthStart Antibody binds *Tth* DNA polymerase with high affinity under the conditions stated in this protocol. **Please note that the use of formamide with TthStart Antibody is not recommended due to interference with TthStart function.**

4. [Optional] In all PCR applications we have tested, we have found that TthStart Antibody works reliably when diluted with enzyme as described in Step IV.B.1.b. However, depending on the primers, target, or *Tth* DNA polymerase you are using, you may be able to obtain satisfactory results using a lower ratio of antibody to polymerase. If this is the case, you can try using two-fold serial dilutions of the working dilution (Step IV.B.1.a). Please note, however, that in most cases TthStart Antibody loses its effectiveness if the ratio of antibody to polymerase is below 20:1.

IV. TthStart Protocol *continued*

B. Dilution of the TthStart Antibody for Immediate Use in PCR

1. Dilution of TthStart Antibody and preparation of PCR master mix

   a. Prepare a working dilution of the TthStart Antibody using the supplied dilution buffer. This dilution will supply enough TthStart Antibody to prepare a PCR master mix for 10 PCR amplifications (50-µl reaction volume).

   4.4 µl TthStart Antibody (2.2 µg/µl; 14 µM)
   17.6 µl dilution buffer
   22.0 µl TthStart Antibody (0.44 µg/µl; 2.8 µM)

   b. Mix the diluted TthStart Antibody with *Tth* DNA polymerase. If prepared as described here, the mixture will be enough for 10 PCR reactions, each using 3 µl of the mixture per reaction.

   22 µl TthStart Antibody working dilution (0.44 µg/µl; 2.8 µM)
   11 µl *Tth* DNA polymerase (2–5 units/µl)
   33 µl total

   Note: This mixture of TthStart Antibody and *Tth* DNA polymerase will freeze at –20°C, but it can be stored at 4°C for up to 3 months for later use. See Section IV.C if you want to prepare aliquots of premixed TthStart and *Tth* DNA polymerase that can be stored at –20°C for later use.

   c. Incubate the mixture of TthStart Antibody and *Tth* DNA polymerase at room temperature (20–22°C) for 5 min before assembling PCR reactions. The TthStart Antibody/*Tth* DNA polymerase mixture can be incubated for up to 30 min with no deleterious effects.

   d. Use a master mix to minimize tube-to-tube variation in PCR. Table I describes PCR master mix I—a typical master mix for 10 PCR reactions, based on a final volume of 50 µl per tube.

   e. Combine your cDNA or DNA sample with PCR master mix I as follows:

   48 µl PCR master mix I
   2 µl cDNA or DNA sample
   50 µl total

   f. Add approximately 50 µl of mineral oil to each tube to prevent evaporation during thermal cycling.

2. PCR thermal cycling

   Begin the DNA thermal cycling program you normally use. Please note that TthStart Antibody may result in greater yields and/or sensitivity; therefore, fewer cycles may be needed to achieve the same yield.

IV. TthStart Protocol *continued*

TABLE I: PCR MASTER MIX I

Reagent	Per rxn (µl)	For 10 rxns (µl) (+ 10% extra)	Final Conc. (µM)*
10X *Tth* reaction buffer	5.0	55.0	1X
20 µM 5' primer	1.0	11.0	0.40
20 µM 3' primer	1.0	11.0	0.40
dNTP mixture [10 mM each dNTP]	1.0	11.0	200 (each)
25 mM $Mg(OAc)_2$	2.2	24.2	1100
dH_2O	34.8	382.8	—
Freshly prepared, mixed TthStart Antibody + *Tth* DNA polymerase	3.0	33.0	
Total volume	48.0	528.0	

* Final concentration of components in the reaction mixture, based on a 50-µl final reaction volume.

C. Preparing, Storing, and Using Aliquots of Premixed TthStart Antibody and *Tth* DNA Polymerase

The concentrated TthStart Antibody may be added directly to an aliquot of *Tth* DNA polymerase. The mixture of TthStart Antibody and *Tth* DNA polymerase may then be aliquoted and stored at –20°C for up to 6 months. This is convenient if you plan to use the same molar ratio of TthStart Antibody to *Tth* DNA polymerase for a number of experiments.

Note: If the mixture is prepared as described in Step 1.a below, the mixture will not freeze when stored at –20°C, due to the high concentration of glycerol (50%, if the polymerase is also in a storage buffer containing 50% glycerol).

1. Mixing, aliquoting, and storing TthStart Antibody/*Tth* DNA polymerase
   a. The example below gives the reagent amounts sufficient for one batch of PCR master mix II (10 PCR amplifications), which is described in Step 2 below. These volumes can be scaled up if you are planning to aliquot and store the premixed TthStart Antibody/ *Tth* DNA polymerase.

      4.4 µl TthStart Antibody (2.2 µg/µl; 14 µM)
      11.0 µl Tth DNA polymerase (2–5 units/µl)
      15.4 µl total mixture

IV. TthStart Protocol *continued*

b. Incubate the mixture of TthStart Antibody and *Tth* DNA polymerase at room temperature (20–22°C) for 5 min before aliquoting and storing or adding it to PCR master mix II (Step 2.a below). The TthStart Antibody/*Tth* DNA polymerase mixture can be incubated for up to 30 min with no deleterious effects.

c. If desired, aliquot the mixture of TthStart Antibody and *Tth* DNA polymerase for use at a later date. Aliquots prepared this way can be stored at –20°C for up to 3 months.

2. Use of premixed TthStart/*Tth* DNA polymerase in PCR

a. Use a master mix to minimize tube-to-tube variation in PCR. The prealiquoted TthStart Antibody/*Tth* DNA polymerase mixture can be added directly to a PCR master mix. Table II describes PCR master mix II—a typical master mix for 10 PCR tubes, based on a final reaction volume of 50 µl per tube. Unlike PCR master mix I (Table I), PCR master mix II uses the premixed TthStart/*Tth* Polymerase prepared above in Section IV.C.1.

TABLE II: PCR MASTER MIX II

Reagent	Per rxn (µl)	For 10 rxns (µl) (+ 10% extra)	Final Conc.(µM)*
10X *Tth* reaction buffer	5.0	55.0	1X
20 µM 5' primer	1.0	11.0	0.40
20 µM 3' primer	1.0	11.0	0.40
dNTP mixture [10 mM each dNTP]	1.0	11.0	200 (each)
25 mM $Mg(OAc)_2$	2.2	24.2	1100
dH_2O	36.4	400.4	—
Concentrated, premixed TthStart Antibody + *Tth* DNA polymerase	1.4	15.4	
Total volume	48.0	528.0	

* Final concentration of components in the reaction mixture, based on a 50-µl final reaction volume.

IV. TthStart Protocol *continued*

b. To assemble PCR reactions, combine your cDNA or DNA sample with PCR master mix II as follows:

48 µl PCR master mix II
2 µl cDNA or DNA sample
50 µl total

c. Add approximately 50 µl of mineral oil to each tube to prevent evaporation during thermal cycling.

d. Begin temperature cycling as described in Step IV.B.2.

V. Troubleshooting Guide

The simplicity of the TthStart Antibody system makes its use fairly straightforward. If the hot start PCR method improves a particular system, it is likely to be improved by the TthStart Antibody. Consequently, if no reduction of nonspecific products is observed when using TthStart Antibody, the first thing to do is to test the PCR system with a conventional hot start method.

A. Both TthStart PCR and the conventional hot start PCR yield multiple nonspecific products.

1. Raise the annealing temperature in 2–3°C increments. Raising the temperature will improve the specificity of binding by the primers, but it may also result in reduced binding and extension of the primers. If raising the annealing temperature causes a reduced yield of the specific product with only a proportional reduction of side reaction products, it may be necessary to redesign the primers (9).
2. Take special precautions to avoid crossover contamination of PCR reactions with both specific and nonspecific PCR products, including primer-dimer artifacts (10).
3. Titration of the TthStart Antibody may be necessary to achieve the same degree of improvement as with a conventional hot start, especially if you are using a modified *Tth* DNA polymerase (see Section IV.A.2) or PCR reaction conditions other than those suggested in this protocol. If you feel such an antibody titration is necessary, try starting with a working dilution (Step IV.B.1) that has a two- to four-fold higher concentration of TthStart Antibody than that recommended in the protocol.

B. Yield of specific product is low using TthStart Antibody.

1. If greater yield is necessary, you may increase the reaction volume to 100 or 150 µl or more so that more of the product can be generated.
2. Increase number of amplification cycles. If you are using 25–30 cycles and obtaining weak yields, increasing cycle number to 35–40 should result in greater yields without significantly increasing side reaction products.
3. Modify reaction conditions and/or selection of PCR targets to obtain greater opportunities for primer annealing. For example, increase the denaturation time up to 1–1.5 min and/or increase the denaturation temperature to as high as 95°C to overcome denaturation difficulties. Extremely GC-rich regions should be avoided as PCR targets, if possible. **Please note that the use of formamide with TthStart Antibody is not recommended, due to interference with TthStart function.** Alternatively, reduce the annealing temperature in 2–3°C increments or increase the Mg^{2+} concentration in 0.5-mM increments to increase yield without additional side reaction products. This approach may be especially helpful with primers which have bases mismatched to the target sequence.

V. Troubleshooting Guide *continued*

4. If TthStart Antibody is used at a concentration 5-fold greater than that recommended in the protocol, there is a risk that the excess antibody or glycerol from the storage buffer may inhibit the reaction. If this is the case, titration of the TthStart Antibody may alleviate the problem.

VI. References

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VII. Related Products

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• Advantage® Genomic PCR Kit	K1906-1,-y
• GenomeWalker™ Kits	many
• TaqStart™ Antibody	5400-1
• Advantage® UltraPure PCR Deoxynucleotide Mix	4700-1

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TaqStart™ Antibody User Manual

PT1576-1 (PR11012)

Published 23 January 2001

Catalog #: 5400-1, -2

See List of Components for storage conditions

FOR RESEARCH USE ONLY

Table of Contents

I. Introduction

"Hot start" PCR is commonly used to enhance the specificity and sensitivity of PCR (D'Aquila *et al.*,1991; Chou *et al.*, 1992; Faloona *et al.*, 1990). In many cases, hot start PCR produces greater yields of single products than has been possible with conventional PCR. However, manual hot start PCR is inconvenient, time-consuming, and incurs a risk of cross-contamination. TaqStart Antibody makes it possible to perform "TaqStart" PCR, with all the advantages—and none of the disadvantages—of hot start PCR.

TaqStart Antibody is a neutralizing monoclonal antibody to *Taq* DNA polymerase (Kellogg *et al.*, 1994; Findlay *et al.*, 1993; Sharkey *et al.*, 1994). TaqStart Antibody is used to block polymerase activity during set-up of the PCR reactions at ambient temperatures (20–22°C). The inhibition of *Taq* DNA polymerase is completely reversed when the temperature is raised above 70°C. At the first template denaturation step in thermal cycling, the enzyme-antibody complex dissociates and the TaqStart Antibody is rendered nonfunctional. At the same time, the activity of *Taq* DNA polymerase is restored, and the enzyme functions normally during the course of the PCR. TaqStart is effective with a variety of commercially available *Taq* DNA polymerases (native and recombinant). TITANIUM™ *Taq* and all of our Advantage® 2 products are formulated with TaqStart Antibody.

The use of TaqStart Antibody significantly improves the results of PCR amplifications which can be improved by a hot start. In many cases, TaqStart PCR has been proven to prevent generation of nonspecific amplification products and primer-dimer artifacts. This means that more definitive PCR results can often be obtained in cases where generation of nonspecific amplification products is a problem. Typical applications for TaqStart Antibody include PCR reactions involving one or more of the following: complex genomic or cDNA templates; very low-copy-number targets; large numbers of thermal cycles (>35); and multiple primer pairs in the same tube (i.e., multiplex PCR).

II. List of Components

The amounts provided are sufficient for 200 or 500 reactions (using 2 units *Taq* DNA polymerase per reaction). Store components at –20°C.

5400-1 (200 reactions):

- **80 µl** **TaqStart Antibody** (7 µM; 1.1 µg/µl) in storage buffer: 50 mM KCl, 10 mM Tris-HCl (pH 7.0), 50% glycerol
- **1.0 ml** **Dilution buffer**: 50 mM KCl, 10 mM Tris-HCl (pH 7.0)

5400-2 (500 reactions):

- **200 µl** **TaqStart Antibody** (7 µM; 1.1 µg/µl) in storage buffer
- **2 x 1.0 ml** **Dilution buffer**

III. Additional Materials Required

Note: The following materials are required but not supplied.

- **10X PCR reaction buffer**

Tris-HCl	100 mM
KCl	500 mM
$MgCl_2$	15 mM
pH 8.3 (at 25°C)	

Note: Variations in the concentration of $MgCl_2$, or the addition of gelatin, should not affect the performance of the TaqStart Antibody.

- **dNTP mixture** [dATP, dCTP, dGTP, & dTTP; 10 mM final concentration of each dNTP in mixture]
- **Sterile, distilled water**
- ***Taq* DNA polymerase**

IV. TaqStart Protocol

PLEASE READ ENTIRE PROTOCOL BEFORE STARTING.

A. General Considerations

1. Storage and use

   In the supplied storage buffer (containing 50% glycerol) and at the supplied concentration (>1 µg/µl), TaqStart Antibody will not freeze at –20°C. However, repeated freezing and thawing of diluted TaqStart Antibody may adversely affect its function. There are two basic approaches to using TaqStart Antibody which avoid this problem:

   a. A portion of the TaqStart Antibody can be diluted to a working concentration and mixed with *Taq* DNA polymerase immediately prior to use, as described in Section IV.B. The diluted portion may be stored at 4°C for ≤3 months. Store the undiluted portion of the TaqStart Antibody at –20°C. This alternative is recommended if you wish to retain the option of using different molar ratios of TaqStart Antibody to *Taq* DNA polymerase for different experiments.

   b. TaqStart Antibody can be added directly (i.e., without first diluting) to a batch of *Taq* DNA polymerase, aliquoted, and stored at –20°C for later use, as described in Section IV.C. This alternative is more convenient than the above alternative if you plan to use the same molar ratio of TaqStart Antibody to *Taq* DNA polymerase for a number of experiments.

2. Choice of enzymes

   TaqStart Antibody has been developed to bind to and inactivate DNA polymerase of *Thermus aquaticus* YT1 strain, and will function well with commercially available *Taq* DNA polymerases licensed for use in PCR, using a molar ratio of 28:1 (antibody:polymerase). DNA polymerases of species other than *T. aquaticus* are not likely to benefit from use of TaqStart. Some genetically altered forms of *Taq* DNA polymerase may have significantly different specific activities, mutated binding sites, or other factors which may require different molar ratios for optimal results. Thus, it may be necessary to titrate the TaqStart Antibody (relative to the polymerase) before you start your experiments (see Section V.B).

3. Use of additives

   TaqStart Antibody binds *Taq* DNA polymerase with high affinity under the conditions stated in this protocol. **Please note that the use of DMSO or formamide with TaqStart Antibody is not recommended due to interference with TaqStart function.** Other cosolvents, solutes (e.g., salts) and extremes of pH or other reaction conditions may reduce the affinity of TaqStart Antibody for the polymerase and thereby compromise the effectiveness of the antibody.

IV. TaqStart Protocol *continued*

4. [Optional] In all the PCR applications we have tested, we have found that TaqStart Antibody works reliably at a molar ratio of 28:1 . However, depending on the primers, target, or *Taq* DNA polymerase you are using, you may be able to obtain satisfactory results using a lower ratio of antibody to polymerase. If this is the case, you can try using two-fold serial dilutions of the working dilution (Step IV.B.1.a). Please note, however, that, in most case, TaqStart Antibody loses its effectiveness if the ratio of antibody to polymerase is below 10:1.

B. Dilution of the TaqStart Antibody for Immediate Use in PCR

1. Dilution of TaqStart Antibody and preparation of PCR master mix

   a. Prepare a working dilution of TaqStart Antibody using the supplied Dilution buffer. The dilution described here will supply enough TaqStart Antibody to prepare a PCR master mix for 10 PCR amplifications (50 µl reaction volume).

      4.4 µl TaqStart Antibody (1.1 µg/µl; 7 µM)
      17.6 µl Dilution buffer
      22.0 µl TaqStart Antibody (0.22 µg/µl; 1.4 µM)

   b. Mix the diluted TaqStart Antibody with *Taq* DNA polymerase. We recommend a dilution of 28 (molar) parts TaqStart Antibody to 1 (molar) part *Taq* DNA polymerase. If prepared as described here (i.e., using a molar ratio of 28:1), the mixture will be sufficient for 10 PCR reactions, each using 2.4 µl of the mixture per reaction.

      22.0 µl TaqStart Antibody working dilution (0.22 µg/µl; 1.4 µM)
      4.4 µl *Taq* DNA polymerase (5 units/µl or 0.25 µM)
      26.4 µl total

      Note: This mixture of TaqStart Antibody and *Taq* DNA polymerase will freeze at –20°C, but it can be stored at 4°C for≤3 months for later use. See Section IV.C if you want to prepare aliquots of premixed TaqStart and *Taq* DNA polymerase that can be stored at –20°C for later use.

   c. Incubate the mixture of TaqStart Antibody and *Taq* DNA polymerase at room temperature (20–22°C) for 5 min before assembling PCR reactions. The TaqStart Antibody/*Taq* DNA polymerase mixture can be incubated for up to 30 min with no deleterious effects.

   d. CLONTECH recommends using a master mix to minimize tube-to-tube variation in PCR. Table I at the top of the next page describes PCR master mix I—a typical master mix for 10 PCR tubes, based on a final reaction volume of 50 µl per tube.

IV. TaqStart Protocol *continued*

TABLE I: PCR MASTER MIX I

Reagent	Per rxn	For 10 rxns (+ 10% extra)	Final Conc.*
10X PCR reaction buffer	5 µl	55 µl	
20 µM 5' primer	1 µl	11 µl	0.40 µM
20 µM 3' primer	1 µl	11 µl	0.40 µM
dNTP mixture [10 mM each dNTP]	1 µl	11 µl	0.20 mM (each)
dH_2O	37.6 µl	413.6 µl	
Freshly prepared 28:1 mixture of TaqStart Antibody + *Taq* DNA polymerase	2.4 µl	26.4 µl	0.056 µM 0.002 µM
Total volume	48 µl	528 µl	

* Final concentration of components in the reaction mixture, based on a 50-µl final reaction volume.

e. Combine your cDNA or DNA sample with PCR master mix I as follows:

48 µl PCR master mix I
2 µl cDNA or DNA sample
50 µl total

f. Add approximately 50 µl of mineral oil to each tube to prevent evaporation during thermal cycling.

2. PCR thermal cycling

Use the DNA thermal cycling program you normally use. Please note, however, that greater yields and/or sensitivity may result from the use of TaqStart Antibody; therefore, fewer temperature cycles may be needed to achieve the same yield. In the case of PCR reactions using extremely low copy-number target sequences, additional cycles (up to a total of 40–45) may be used to generate enough product to visualize by ethidium bromide staining after gel electrophoresis.

IV. TaqStart Protocol *continued*

C. Preparing, Storing, and Using Aliquots of Premixed TaqStart Antibody and *Taq* DNA Polymerase

The concentrated TaqStart Antibody may be added directly to an aliquot of *Taq* DNA polymerase. The mixture of TaqStart Antibody and *Taq* DNA polymerase may then be aliquoted and stored at –20°C for up to 6 months. This is convenient if you plan to use the same molar ratio of TaqStart Antibody to *Taq* DNA polymerase for a number of experiments.

Note: If the mixture is prepared as described in Step 1.a below, the mixture will not freeze when stored at –20°C, due to the high concentration of glycerol (50%, if the polymerase is also in a storage buffer containing 50% glycerol).

1. Mixing, aliquoting, and storing TaqStart Antibody/*Taq* DNA polymerase
   a. Add one volume of TaqStart Antibody to one volume of *Taq* DNA polymerase. The example below gives the reagent amounts sufficient for 1 batch of PCR master mix II (10 PCR amplifications + 10% extra), which is described in Step 2 below. These volumes can be scaled up if you are planning to aliquot and store the premixed TaqStart Antibody/*Taq* DNA polymerase.

      4.4 µl TaqStart Antibody (1.1 µg/µl; 7 µM)

      4.4 µl *Taq* DNA polymerase (5 units/µl; 0.25 µM)

      8.8 µl total mixture

   b. Incubate the mixture of TaqStart Antibody and *Taq* DNA polymerase at room temperature (20–22°C) for 5 min before aliquoting and storing or adding it to PCR master mix II (Step 2.a below). The TaqStart Antibody/*Taq* DNA polymerase mixture can be incubated for up to 30 min with no deleterious effects.
   c. If desired, aliquot the mixture of TaqStart Antibody and *Taq* DNA polymerase for use at a later date. Aliquots prepared this way can be stored at –20°C for up to 3 months.
2. Use of premixed TaqStart/*Taq* DNA polymerase in PCR
   a. CLONTECH recommends using a master mix to minimize tube-to-tube variation in PCR. The prealiquoted TaqStart Antibody/*Taq* DNA polymerase mixture can be added directly to a PCR master mix. Table II at the top of the next page describes PCR master mix II—a typical master mix for 10 PCR tubes, based on a final reaction volume of 50 µl per tube. Unlike PCR master mix I (Table I), PCR master mix II uses the premixed TaqStart/*Taq* Polymerase prepared above in Section IV.C.1.

IV. TaqStart Protocol *continued*

TABLE II: PCR MASTER MIX II

Reagent	Per rxn	For 10 rxns (+ 10% extra)	Final Conc.*
10X PCR reaction buffer	5 µl	55 µl	
20 µM 5' primer	1 µl	11 µl	0.40 µM
20 µM 3' primer	1 µl	11 µl	0.40 µM
dNTP mixture [10 mM each dNTP]	1 µl	11 µl	0.20 mM (each)
dHO	39.2 µl	431.2 µl	
Concentrated, premixed TaqStart Antibody + *Taq* DNA polymerase	0.8 µl	8.8 µl	0.056 µM 0.002 µM
Total volume	48 µl	528 µl	

* Final concentration of components in the reaction mixture, based on a 50-µl final reaction volume.

b. To assemble PCR reactions, combine your cDNA or DNA sample with PCR master mix II as follows:

48 µl PCR master mix II
2 µl cDNA or DNA sample
50 µl total

c. Add approximately 50 µl of mineral oil to each tube to prevent evaporation during thermal cycling.

d. Commence temperature cycling as described in Step IV.B.2.

V. Troubleshooting Guide

The simplicity of the TaqStart Antibody system makes its use fairly straightforward. If a particular system is helped by the hot start PCR method, it is likely to be helped by the TaqStart Antibody. Consequently, if no reduction of nonspecific products is observed when using TaqStart Antibody, the first thing to do is to test the PCR system with a conventional hot start method.

A. Both TaqStart PCR and the Conventional Hot Start PCR Yield Multiple Nonspecific Products.

1. Raise the annealing temperature in 2–3°C increments. Raising the temperature will improve the specificity of binding by the primers, but it may also result in reduced binding and extension of the primers. If raising the annealing temperature causes a reduced yield of the specific product with only a proportional reduction of side reaction products, it may be necessary to redesign the primers (Huang & Jeang, 1994)).
2. Take special precautions to avoid crossover contamination of PCR reactions with both specific and nonspecific PCR products, including primer-dimer artifacts (Kwok & Higuchi, 1989).

B. TaqStart PCR Yields More Nonspecific Products Than Conventional Hot Start PCR.

Titration of the TaqStart Antibody may be necessary to achieve the same degree of improvement as with a conventional hot start, especially if you are using a modified *Taq* DNA polymerase (see Section IV.A.2) or if you are using PCR reaction conditions other than those suggested in this protocol. If you feel such an antibody titration is necessary, we suggest starting with a working dilution (Step IV.B.1) that has a two- to four-fold higher concentration of TaqStart Antibody than that recommended in the protocol.

C. Yield of Specific Product is Low Using TaqStart Antibody.

1. If greater yield is necessary, you may increase the reaction volume to 100 or 150 µl or more so that more of the product can be generated.
2. Increase number of amplification cycles. If you are using 25–30 cycles and obtaining weak yields, increasing cycle number to 35–40 should result in greater yields without significantly increasing side reaction products.
3. Modify reaction conditions and/or selection of PCR targets to obtain greater opportunities for primer annealing. For example, increase the denaturation time up to 1–1.5 min and/or increase the denaturation temperature to as high as 95°C to overcome denaturation difficulties. Extremely GC-rich regions should be avoided as PCR targets, if possible. **Please note that the use of DMSO or formamide with TaqStart Antibody is not recommended, due to interference with TaqStart function.** Alternatively, reduce the annealing temperature in

V. Troubleshooting Guide *continued*

2–3°C increments or increase the $MgCl_2$ concentration in 0.5-mM increments to increase yield without additional side reaction products. This approach may be especially helpful with primers which have bases mismatched to the target sequence.

4. Increasing the pH of the reaction in 0.3-unit increments (to a maximum of pH 9.2) or increasing the concentration of KCl to 75 mM may increase the yield of specific PCR products in some cases.
5. If TaqStart Antibody is used at a concentration greater than 5-fold more than that recommended in the protocol, there is a risk that the excess antibody or glycerol from the storage buffer may inhibit the reaction. If this is the case, titration of the TaqStart Antibody may help to alleviate the problem.

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Notes

Notes